Kif1C, a kinesin-like motor protein, mediates mouse macrophage resistance to anthrax lethal factor

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Background: Inbred mouse strains exhibit striking differences in the susceptibility of their macrophages to the effects of anthrax lethal toxin (LeTx). Previous data has shown that this difference in susceptibility lies downstream of toxin entry into macrophages. A locus controlling this phenotype, called *Ltxs1*, has been mapped to chromosome 11, but the responsible gene has not been identified.

Results: Here, we report the identification of the *Ltxs1* gene as *Kif1C*, which encodes a kinesin-like motor protein of the UNC104 subfamily. *Kif1C* is the only gene in the *Ltxs1* interval exhibiting polymorphisms between susceptible and resistant strains. Multiple alleles of *Kif1C* determine the susceptibility or resistance of cultured mouse macrophages to LeTx. Treatment of resistant macrophages with brefeldin-A (which alters the cellular localization of Kif1C) induces susceptibility to LeTx, while ectopic expression of a resistance allele of *Kif1C* in susceptible macrophages causes a 4-fold increase in the number of cells surviving LeTx treatment. We also show that cleavage of map kinase kinase 3, a target of LeTx proteolysis, occurs in resistant cells.

Conclusions: We conclude that mutations in *Kif1C* are responsible for the differences in the susceptibility of inbred mouse macrophages to LeTx and that proper Kif1C function is required for LeTx resistance. Since the LeTx-mediated proteolysis of map kinase kinase 3 occurs even in resistant cells, Kif1C does not affect cellular entry or processing of LeTx and likely influences events occurring later in the intoxication pathway.

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Background

The lethal effects of systemic anthrax can be mimicked in animal models by the administration of anthrax lethal toxin (LeTx), which is produced at high levels during systemic infection [1]. LeTx is made up of two polypeptides: protective antigen (PA) and lethal factor (LF) [2]. PA mediates the delivery of LF into the cytosol of all cell types that have been tested, while LF exerts a cytolytic effect that is specific to macrophages [2, 3].

The cytolysis of macrophages that is induced by LF intoxication most closely resembles necrosis (rather than apoptosis) and is perhaps a response to the overproduction of or inappropriately contained exposure to cytokines or reactive oxygen intermediates [4]. The toxic activity of LF depends on its Zn^{2+} metallopeptidase activity [5, 6], strongly suggesting that proteolysis of one or more cellular proteins unleashes a cascade of events that results in the death of the intoxicated macrophage. In support of this model, LF cleaves several distinct mitogen-activated protein kinase kinase (MAPKK) species; however, the physiological importance of this MAPKK proteolysis relative to macrophage cytolysis has not been established [7, 8].

Inbred mouse strains exhibit striking differences in the susceptibility of their cultured macrophages to the effects of LeTx. Genetic differences between a susceptible strain (C3H/HeJ) and a resistant strain (C57BL/6J) have been exploited to map a single gene (named *Ltxs1*) that controls this phenotype to chromosome 11 [9, 10]. In an attempt to understand the mechanism of LeTx-induced macrophage cytolysis, we pursued a positional cloning strategy to identify the *Ltxs1* gene.

Here, we report the identification of the *Ltxs1* gene as *Kif1C*, which encodes a kinesin-like motor protein of the UNC104 subfamily. We show that *Kif1C* is the only gene in the *Ltxs1* critical interval that contains mutations between susceptible and resistant inbred strains. Treatments that alter the cellular localization of Kif1C induce susceptibility in normally resistant macrophages, and ectopic expression of a resistance allele of *Kif1C* rescues normally susceptible cells. We also show that LF exhibits proteolytic activity in both resistant and susceptible cells. Our data demonstrate that Kif1C is not involved in toxin uptake or activation and that a high level of Kif1C function is required for macrophages to survive LeTx treatment.

Results

Identification of candidate genes

In order to identify all positional candidate genes, we generated a virtually complete genomic sequence for the mouse *Ltxs1* interval using BAC clones derived from strains C57BL/6J and 129S3 (129S3 is a susceptible strain in which the LeTx susceptibility phenotype maps to the *Ltxs1* interval [10]). To aid in the identification of candidate genes, this mouse sequence was compared to the publicly available genomic sequence data of the syntenic human interval. As shown in Figure 1a, the structure of the mouse and human regions are very similar, with a few notable exceptions. First, there is a break in synteny homology in the middle of the interval, in which three human genes are present, but no homologous mouse genes can be detected. Additionally, the two most distal genes, XM_012675 and KIAA0926, appear to be nonfunctional in the mouse. Comparative sequence analysis revealed that the first three exons of KIAA0926, as well as the coding region of XM_012675, are not present in the genome of strain 129S3, while RT-PCR and Northern blot analysis failed to detect the expression of XM_012675 or KIAA0926 in susceptible or resistant mouse macrophages (data not shown). Furthermore, BLAST searches revealed that there are no mouse ESTs homologous to either XM_012675 or KIAA0926, indicating that these two genes are likely not expressed in the mouse.

XM_012675 and KIAA0926 are present in an area of the mouse genome that appears to have undergone a repetitive expansion, with at least three partial copies of KIAA0926 being present in strain 129S3 (see Figure 1b). We have previously shown that different inbred strains contain expansions of variable size at this location [10]. The possibility that this genomic expansion represents the *Ltxs1* mutation can be ruled out for two reasons: first, the presence or absence of this expansion does not perfectly correlate with LeTx susceptibility [10], and, second, LeTx susceptibility maps to the *Ltxs1* interval in a recombinant inbred strain panel derived from strains C57L/J and AKR/J [9], two strains that have opposite phenotypes but an identical expansion [10].

Identification of mutations in Kif1C

Analysis of the expression patterns and coding sequence of all candidate genes in the *Ltxs1* interval revealed that only one gene, *Kif1C*, exhibited any polymorphisms between resistant and susceptible strains. *Kif1C* is a ubiquitously expressed gene encoding an 1100-amino acid kinesin-like motor protein of the UNC104 subfamily that is likely to be involved in the intracellular transport of molecular cargo [11]. The human homolog of Kif1C has been implicated in retrograde vesicular transport from the Golgi apparatus to the Endoplasmic Reticulum [12]. The exact molecular nature of the cargo of Kif1C, however, remains unknown.

Despite this ambiguity, Kif1C contains a number of domains that are likely to be important for its function (see Figure 2a). Just C-terminal to the conserved kinesin motor domain is a forkhead homology-associated (FHA) domain, a feature shared by all kinesin UNC104 subfamily members [12]. It has been shown that the human homolog of Kif1C binds the phosphatase PTPD1 and that human Kif1C interacts with 14-3-3 proteins in a phosphorylation-dependent manner. Additionally, there is a short stretch of amino acids that is predicted to form a coiled-coil domain, which may participate in homodimerization [13].

We see a C → T transition in *Kif1C* that results in a proline to leucine substitution at position 578 in the susceptible strain C3H/HeJ relative to the resistant strain C57BL/6J. This substitution alters an evolutionarily conserved proline in the forkhead homology-associated (FHA) domain, which is thought to be involved in protein-protein interactions regulated by phosphorylation [12, 14]. This evolutionary conservation suggests that the proline at position 578 is functionally important in Kif1C and that the substitution of this amino acid is likely to have a deleterious effect on the function of Kif1C.

In an attempt to correlate the *Kif1C* genotype with the LeTx susceptibility phenotype, we then sequenced the coding region of *Kif1C* in 16 other inbred strains. The $P^{578} \rightarrow L^{578}$ substitution observed between C57BL/6J and C3H/HeJ was by far the most common polymorphism encountered, with 12 other strains exhibiting either the C57BL/6J or C3H/HeJ allele of *Kif1C*. Susceptibility and resistance correlates with L^{578} and P^{578}, respectively, in all 12 strains (see Figure 2b).

We also discovered other alleles of *Kif1C*. These additional alleles all contained the P^{578} observed in the resistant strain C57BL/6J, with various C-terminal amino acid substitutions. Surprisingly, one of these alleles was discovered in strain CAST/Ei, which has the susceptible phenotype. In addition to P^{578}, the CAST/Ei allele of *Kif1C* contains a T → C transition that causes the substitution of P^{1027} for S^{1027}. This mutation apparently confers susceptibility, as confirmed by the fact that the LeTx susceptibility phenotype of CAST/Ei indeed maps to the *Ltxs1* interval (see Materials and methods).

In addition, we identified three other resistant strains (DBA/2J, SM/J, and SPRET/Ei) that have the P^{578} and P^{1027} polymorphisms seen in the CAST/Ei allele of *Kif1C*, with an additional AG → TA nucleotide change introducing a $S^{1066} \rightarrow Y^{1066}$ substitution. Since we have not genetically mapped the LeTx resistance phenotype of strains DBA/2J, SM/J, and SPRET/Ei, it is formally possible that these strains are resistant because of another gene, and the $S^{1066} \rightarrow Y^{1066}$ substitution observed in Kif1C therefore has no functional effect. While this hypothesis needs to be tested,

Figure 1

Genomic comparison of the mouse *Ltxs1* interval and the syntenic human region. **(a)** A gene map of the mouse and human regions. The mouse *Ltxs1* interval is covered by draft-quality sequence (≥4.6× sequence coverage, with a cumulative Phred score of ≥20 for each base). Genes are represented by their accepted abbreviations. In cases where no accepted abbreviation exists, GenBank accession numbers of transcripts homologous to the identified gene are listed. The centromere is oriented toward the left of the figure, and an arrow represents the direction of gene transcription. *Kif1C* is indicated in bold. The genetic markers that flank the *Ltxs1* region are shown inside vertical rectangles [10]. The horizontal bars beneath the genes represent BAC clones (listed by the accession number of their available sequences) that were used to construct the ordered and oriented sequence constructs depicted in **(b)**. The dashed portion of mouse BAC AC090293 was not included in the ordered and oriented mouse sequence construct. (b) A dot-plot, generated using PipMaker [27], showing the relative homology between the human and mouse sequence constructs. The human interval is displayed left to right, and the mouse interval is displayed bottom to top; the lengths of the intervals are indicated in base pairs. An area of homology is indicated by a dot at the corresponding coordinate. Hash marks along the bottom of the plot represent sequence gaps in the human assembly. Landmark genes are indicated according to their position. The region of no mouse homology and the mouse expansion are indicated within rectangles at their respective positions. The mouse expansion appears to be repetitive, as multiple contiguous regions of the strain 129S3 genome are homologous to one stretch of the human genomic assembly.

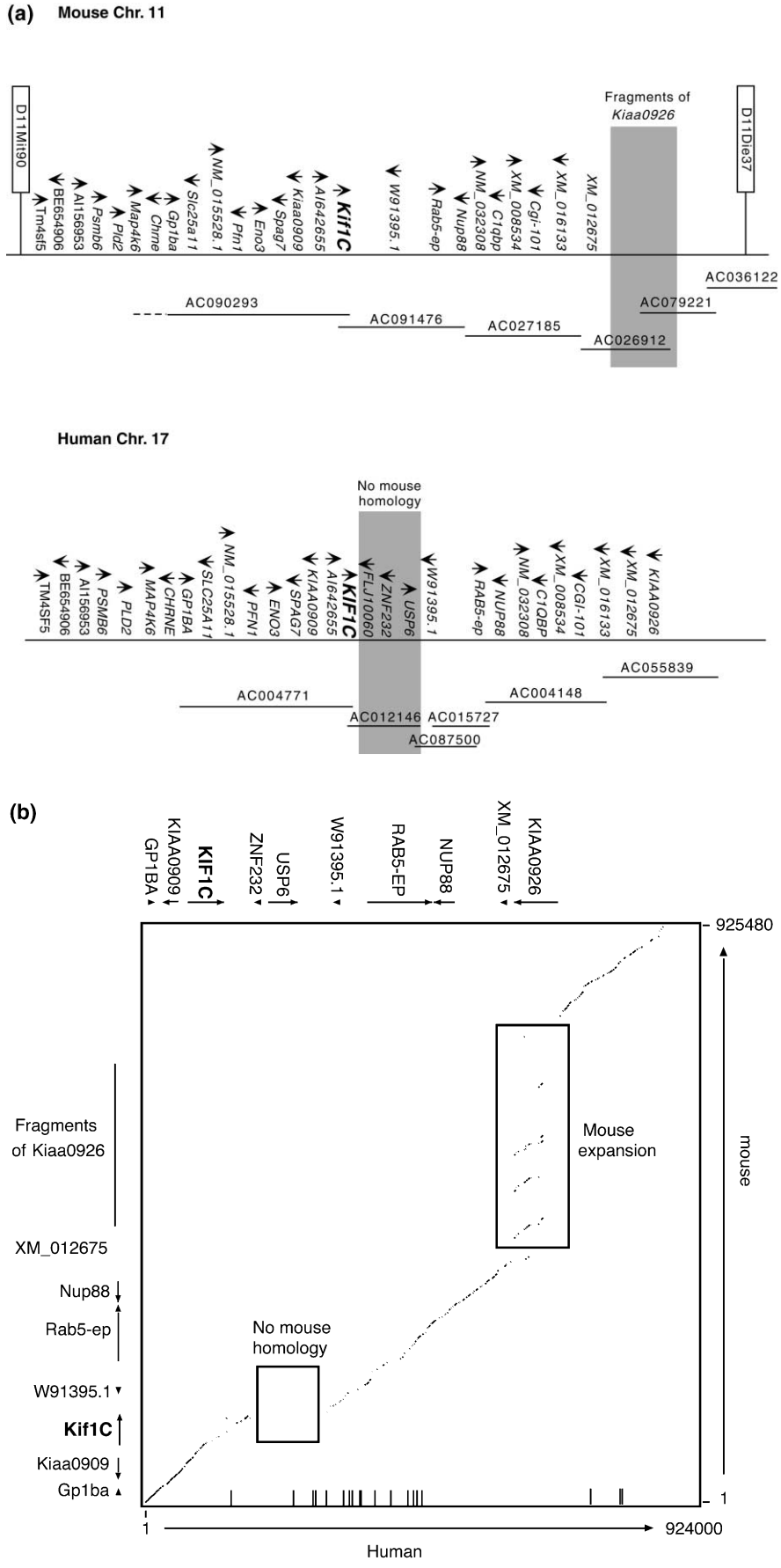
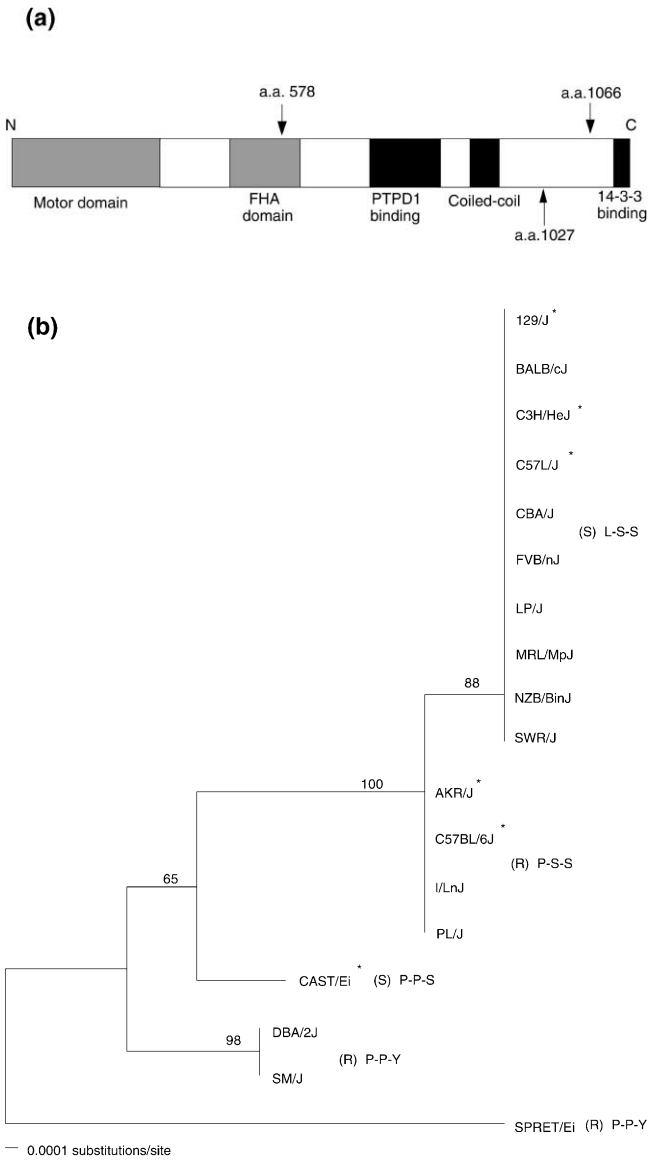


Figure 2



Mutations encountered in Kif1C. **(a)** A schematic representation of mouse Kif1C. Predicted conserved domains are shown as gray boxes. The black boxes represent additional regions identified in the human homolog of Kif1C [13]. The arrows show the positions of the observed missense mutations. **(b)** A neighbor-joining, distance-based phylogram of the *Kif1C* sequence. Strain names are represented at their position on the phylogram, with the corresponding LeTx phenotype shown in parentheses. S = susceptible, R = resistant. Indicated bootstrap values were obtained with 1000 pseudoreplicates. The combination of amino acids at positions 578, 1027, and 1066 of Kif1C is shown to the right of the phenotype for each group. An asterisk denotes a strain for which the LeTx susceptibility phenotype has been mapped to the *Ltxs1* interval. A parsimony-based method of tree building resulted in an identical tree (data not shown).

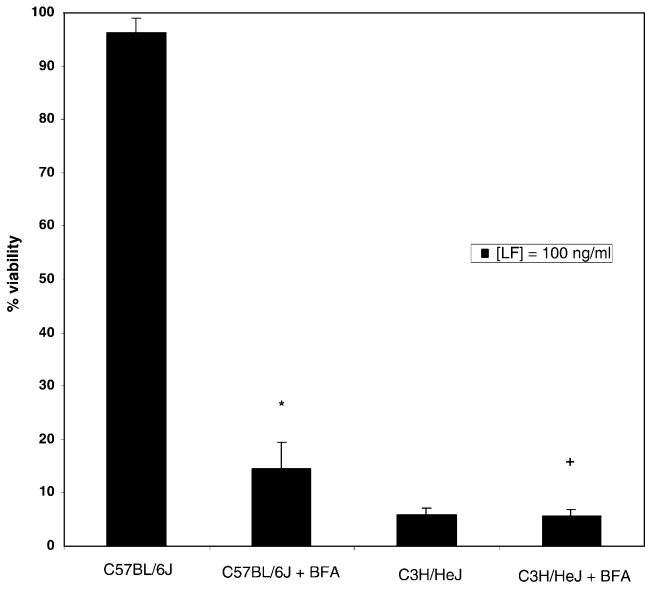
we believe that it is likely that the $S^{1066} \rightarrow Y^{1066}$ substitution observed in Kif1C has functional consequence, as this substitution is only seen in resistant strains (see Figure 2b).

The neighbor-joining, distance-based phylogram shown in Figure 2b demonstrates the molecular relatedness of the various *Kif1C* alleles. The basic architecture of this tree, which separates the commonly used inbred strains from the haplotypes seen in CAST/Ei, DBA/2J, SM/J, and SPRET/Ei, is maintained using sequences throughout the entire *Ltxs1* interval (data not shown). The phylogram shows that susceptible and resistant strains always cluster in the same group according to *Kif1C* sequence and that susceptibility and resistance can be perfectly correlated with the particular combination of amino acids at positions 578, 1027, and 1066. This result suggests that the observed *Kif1C* mutations are causative and are not simply in linkage disequilibrium with the true mutation(s), as this would require that the observed *Kif1C* mutations happened to arise on distinct resistance and susceptibility haplotypes in a manner that perfectly correlates with the phenotype. Although positions 1027 and 1066 are not in evolutionarily conserved domains, they are in the C-terminal tail region of Kif1C that is thought to participate in cargo binding. While the regulation of motility and cargo binding is poorly understood, even minor changes in the C-terminal tail domain have been shown to greatly affect the performance of kinesin in vivo [15]. It is therefore likely that mutations in this region affect the ability of Kif1C to bind or transport molecular cargo.

Disruption of Kif1C localization induces susceptibility

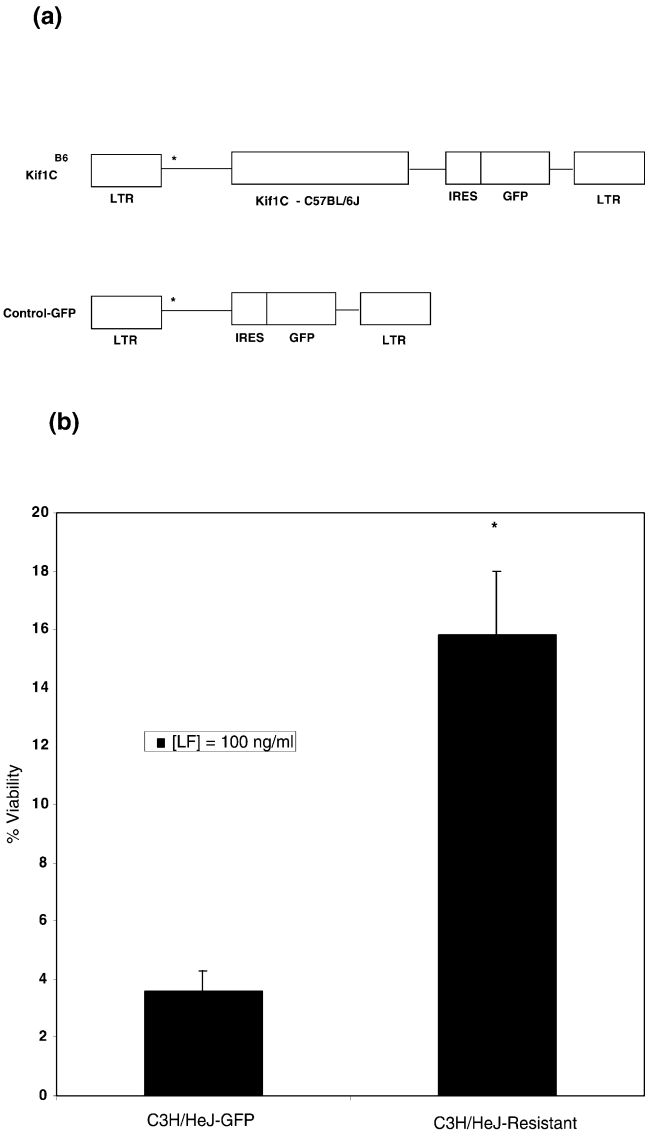
It has previously been reported that the majority of Kif1C localizes to the Golgi apparatus in NIH3T3 cells and that treatment with the fungal metabolite brefeldin A (BFA) results in a dramatic relocalization of Kif1C to the Endoplasmic Reticulum [12]. Reasoning that perturbing the cellular localization of Kif1C would alter its ability to perform its molecular function, we challenged C57BL/6J and C3H/HeJ primary bone marrow-derived macrophages with LeTx in the presence of BFA. As shown in Figure 3, intoxication of the cells in the presence of BFA caused the resistant C57BL/6J macrophages to become completely susceptible to intoxication with LF, whereas the susceptibility of C3H/HeJ macrophages was unaltered. This is in accordance with previous studies showing that the susceptible macrophage cell line J774A.1 remains susceptible after treatment with BFA [16]. We also performed the experiment using a double mutant PA (K397D, D425K; kind gift of Dr. John Collier, Harvard Medical School), which has been shown to prevent the translocation of LF into the cytosol of target cells [17]. The double mutant PA completely blocked the susceptibility-inducing effect of BFA (data not shown), demonstrating that this effect of BFA is dependent upon proper internalization and targeting of LF to the cytosol. This is consistent with previous data showing that the genetic difference in susceptibility to LeTx between inbred mouse strains lies downstream of LF entry to the cytosol of target cells [9, 16, 18].

Figure 3



Incubation with BFA causes resistant macrophages to become susceptible to the effects of LeTx. BFA alone had no effect on the viability of either resistant or susceptible cells (data not shown). Data represents the average of four independent experiments ± standard error of the mean (SEM). An asterisk denotes a significant difference from non-BFA-treated C57BL/6J cells, $p < .0001$. A plus sign denotes a nonsignificant difference from non-BFA-treated C3H/HeJ cells, $p > .25$.

Figure 4



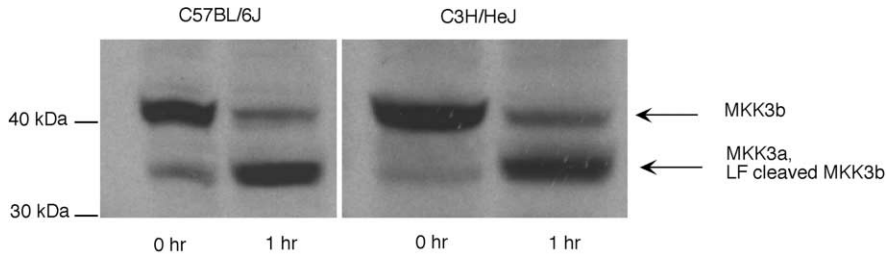
Expression of a resistance allele of Kif1C partially rescues susceptible primary macrophages from LeTx-induced cytolysis. **(a)** A schematic representation of the retroviral constructs containing the C57BL/6J allele of *Kif1C* ($Kif1C^{B6}$) or GFP only (Control-GFP). The asterisk denotes the retroviral packaging signal. **(b)** Rescue of susceptible macrophages. Susceptible primary macrophages were transduced with the control-GFP construct (C3H/HeJ-GFP) or the $Kif1C^{B6}$ construct (C3H/HeJ-Resistant), and viability in response to LeTx was determined. Data represents the average of two independent experiments ± standard error of the mean (SEM). An asterisk denotes a significant difference from control-GFP-transduced cells, $p < 0.01$.

Ectopic expression of a resistance allele of Kif1C partially rescues susceptible macrophages

Our data suggest that perturbations of Kif1C function cause macrophages to become susceptible to the cytolytic effects of LeTx. Furthermore, it has been previously observed that F1 hybrids between C57BL/6J and C3H/HeJ are completely susceptible to LeTx, even though they harbor one resistant *Kif1C* allele [10]. Together, these observations imply that the heterozygotes are susceptible due to haploinsufficiency of wild-type Kif1C function. Under this model, any of the observed amino acid substitutions in the susceptible strains would impair the ability of Kif1C to act properly as a molecular motor, and heterozygotes would have a level of Kif1C function that is below a certain threshold required to maintain viability during LeTx challenge.

To test this directly, and to confirm the role of *Kif1C* in resistance to LeTx, we transduced the resistant C57BL/6J allele of *Kif1C* into susceptible C3H/HeJ macrophages using a retroviral vector derived from the moloney murine leukemia virus (MMLV) (Figure 4a). As shown in Figure 4b, expression of the C57BL/6J allele of *Kif1C* resulted in a 4-fold increase in the proportion of C3H/HeJ macrophages that survived LeTx challenge. We believe that this incomplete rescue likely resulted from variable levels of expression of the construct in the primary cells and that the cells that did not survive were expressing a level of $Kif1C^{B6}$ that is below the required resistance threshold. The reciprocal experiment was also performed, in which C57BL/6J macrophages transduced with the C3H/HeJ

Figure 5



LF is active in resistant and susceptible macrophages. C57BL6/J (resistant) and C3H/HeJ (susceptible) macrophages were either untreated (0 hr) or incubated for 1 hr in the presence of anthrax LeTx (1 hr). Protein extracts were separated by SDS-PAGE, blotted, and probed with a polyclonal antibody specific for the C terminus of MKK3 (see Materials and methods). A downward shift of the MKK3b isoform reveals proteolysis by LF.

allele of *Kif1C* were treated in an identical fashion, and these macrophages showed >95% viability in all fields analyzed (data not shown).

Kif1C is not involved in LeTx cellular uptake

We considered the possibility that Kif1C is involved in the process of cellular uptake or activation of LF and that differences in these processes caused by mutations in Kif1C could be the mechanism by which resistant macrophages survive LeTx intoxication. To address this, we assayed the ability of LF to cleave map kinase kinase 3 (MKK3), a known target of LF proteolysis, in resistant and susceptible macrophages. As shown in Figure 5, the anti-MKK3 antibody detected two distinct bands: a higher molecular weight band corresponding to isoform MKK3b and a lower molecular weight band corresponding to isoform MKK3a [19]. After a 1-hr incubation in LeTx, the lower molecular weight band becomes much more prominent in both resistant and susceptible macrophages. This banding pattern is consistent with that seen in previous demonstrations of MKK3 cleavage by LF, in which MKK3b was cleaved but MKK3a was not [8]. The same study also demonstrated that, upon cleavage, isoform MKK3b migrates near the same apparent molecular weight as MKK3a. Therefore, the observed LeTx-induced increase in the relative intensity of the lower molecular weight band demonstrates LF cleavage of MKK3b. Since LF is able to enter resistant cells and cleave a known target of proteolysis, resistant alleles of *Kif1C* cannot be responsible for a loss of the ability to internalize or activate LF.

Discussion

We have shown that the Kif1C molecular motor is an important mediator of macrophage resistance to intoxication with anthrax lethal factor. Based on our current understanding, it seems unlikely that Kif1C is a direct target of LF proteolysis, as the polymorphisms affecting resistance are found in several different places in the primary amino acid sequence, and none of these regions bears any resemblance to the accepted canonical LF recognition sequence [6, 8]. However, because the sites cleaved by LF can be quite distinct, it will be important to validate this hypothesis with additional experiments.

We have also shown for the first time that LF exhibits a map kinase kinase cleavage activity in resistant macrophages. LF cleaved MKK3 in both resistant and susceptible cells, demonstrating that LF is able to enter the cytosol and function in resistant macrophages. Despite the formal possibility of the existence of secondary toxin trafficking pathways potentially affected by Kif1C polymorphisms, years of investigation on LF internalization have revealed no independent evidence for such pathways [2]. Therefore, we consider it very unlikely that Kif1C participates in the cellular uptake or activation of LF. This is supported by several independent observations that the macrophage resistance of certain mouse strains to LeTx is not due to defects in toxin uptake [9, 16, 18]. Based on this reasoning, we believe that Kif1C impacts important physiologic events that are downstream from the initiating LF proteolytic event(s).

The specificity of LeTx for killing macrophages cannot be currently explained by macrophage specificity of any of its known proteolytic targets or downstream effectors. This suggests that LeTx treatment induces a response unique to macrophages, such as a massive inflammatory burst [20] (perhaps influenced by the MAPKK cleavage activity attributed to LF) and that Kif1C is required to function at very high levels in order to accommodate this stress. By this logic, the observed susceptibility alleles of *Kif1C* represent hypomorphs, and the reduced Kif1C function in susceptible and heterozygous macrophages renders them unable to protect themselves from the LF-induced hyperinflammatory response. Resistant macrophages, which harbor two fully functional *Kif1C* alleles, are able to survive this response. It is therefore likely that small kinetic defects in the ability of Kif1C to perform its normal function become deadly upon LeTx treatment. Interestingly, haploinsufficiency of Kif1Bβ, another UNC-104 kinesin family member, has recently been shown to cause Charcot-Marie-tooth disease type 2A in humans [21], supporting the notion that modest reduction of kinesin function can produce deleterious effects in situations in which the cellular transport machinery is stretched to its limits. It is evident that further analysis of Kif1C mutants will provide a clearer picture of the normal func-

tion of Kif1C and its interaction with the LeTx cytolytic pathway.

Animal species differ in their susceptibility both to infection by *Bacillus anthracis* and to anthrax toxin [1], and it is possible that differences in homologous *Kif1C* alleles are responsible for these variations in susceptibility. All publicly available rat and human KIF1C sequences contain amino acids P-P-Y, respectively, at positions homologous to 578, 1027, and 1066 in mouse Kif1C. While this suggests that rat and human macrophages should be resistant to the effects of LeTx, this contradicts previous data demonstrating the susceptibility of primary human and rat macrophages [22, 23]. However, there are many other sites of missense polymorphism between mouse, rat, and human Kif1C (data not shown). Since even subtle disruption of Kif1C function can have profound effects on macrophage susceptibility to LeTx, these polymorphisms must be considered as possible sources of rat and human susceptibility. It is also possible that the susceptibility or resistance of macrophages from other species is due to genes other than *Kif1C*.

The manifestation of anthrax is a complex process, and the outcome of anthrax infection depends on many complex host-pathogen interactions [1, 2, 24]. While LeTx-induced cytolysis of macrophages plays an important role in the overall outcome of anthrax infection, the susceptibility of macrophages in vitro does not always correlate with in vivo susceptibility to infection with virulent or attenuated strains of *B. anthracis* [1, 24]. Nevertheless, the identification of a gene that affects macrophage susceptibility to LeTx provides valuable insight into a poorly understood aspect of anthrax pathogenesis.

Conclusions

We have shown that mutations in Kif1C, a kinesin-like motor protein of the UNC104 subfamily, are responsible for differences in mouse macrophage susceptibility to anthrax LeTx. We have also shown that two fully functional alleles of *Kif1C* are required for macrophage resistance to LeTx, while disruptions of Kif1C function induce LeTx susceptibility. In addition, we have shown that LF is proteolytically active in the cytosol of resistant macrophages, demonstrating that Kif1C is not involved in LeTx uptake or activation. We suggest that Kif1C functions downstream in the intoxication pathway and serves to protect the macrophage from signaling events induced by LF proteolysis of target proteins.

Materials and methods

Macrophage culture

Primary macrophages were differentiated from bone marrow cells following a published protocol [25]. Femurs were flushed with bone marrow media (BMM: RPMI Medium 1640 containing 20% heat-inactivated fetal bovine serum, 30% L-cell-conditioned media as a source of granulocyte/macrophage colony-stimulating factor [9], 200 μM L-glutamine, 10 μg/ml streptomycin, and 10 U/ml penicillin) to obtain approximately 4×10^6 cells. These cells were then cultured in BMM for 6–7 days in 100-mm polystyrene petri dishes that allow weak adherence of macrophages and easy harvesting by scraping.

Genomic sequencing

We have previously reported the generation of 1.1× sequence coverage of the *Ltxs1* interval [10]. In order to generate the complete genomic sequence of the *Ltxs1* interval, we sequenced the following BAC clones, listed by accession number of their available sequence, to a sequence coverage of ≥4.6×, with a cumulative Phred score of ≥20 for each base: AC013775, AC090293, AC091476, AC027185, AC026912, AC079221, and AC036122. The BAC clones AC091476, AC027185, AC026912, and AC079221 were obtained from the CITB BAC library (Research Genetics; derived from strain 129S3), whereas the BAC clones AC013775, AC090293, and AC036122 were obtained from the RPCI-23 BAC library (Research Genetics; derived from strain C57BL/6J). Sequencing of these BAC clones was performed using a shotgun sequencing strategy. BAC DNA isolation, library construction, template preparations, sequencing reactions, and sequence assembly was done as described in [26]. Dot-plots comparing the mouse and human syntenic intervals were generated using PipMaker (http://bio.cse.psu.edu/pipmaker/) [27].

Transcript mapping

In order to identify all candidate genes, our mouse genomic sequence was compared to the near-complete genomic sequence of the syntenic human interval. In addition to BLAST searches and comparison to public and private genome annotations, the gene-finding program GENSCAN (http://genes.mit.edu/GENSCAN) was used to identify genes in the mouse and human regions. PCR assays and oligo probes were developed from the 5′ and 3′ ends of any genes or ESTs that were predicted by GENSCAN and any transcripts to which our genomic sequence showed ≥90% sequence identity over a length of at least 100 bp. The presence or absence of these transcripts was then confirmed by PCR amplification of BAC DNA or hybridization to BAC Southern blots. All BAC Southern blots were performed as previously described [10].

Some gene homologies were detected that are not shown in Figure 1a. Three genes annotated in the Celera (http://www.celera.com) mouse genome assembly of the *Ltxs1* region have been excluded due to their homology to repetitive elements or their lack of homology to any sequence in available public databases. The Celera ID of these genes are: mCG66014, mCG57736, and mCG21191. One gene annotated in the Celera human genome assembly was excluded because it is not annotated in the publicly available human genome assembly and it is not present in the mouse *Ltxs1* interval. The Celera ID of this gene is hCG1643917. In addition, an intronless pseudogene of the mouse ribosomal protein L37A was detected proximal to *Rab5-ep*, but is not included due to its presumed lack of function. Finally, numerous ESTs were not included on the map due to their homology to interspersed repetitive elements, or because they encoded ORFs of less than 140 base pairs. Nevertheless, these ESTs were analyzed for expression or sequence differences between resistant and susceptible mouse strains, and no differences were found. The accession numbers of these mouse ESTs are: AI415455, AK018106, AA797627, NM_014922, NM_021730, AA756428, and AA050425.

Analysis of candidate genes

The expression of all candidate genes was analyzed by RT-PCR and Northern blot analysis between the resistant strain C57BL/6J and the susceptible strain C3H/HeJ. Total macrophage RNA was extracted from primary macrophages using the Rneasy Midi kit (Qiagen). RT-PCR was performed using the SuperScript One-Step RT-PCR system (GIBCO BRL) using 100 ng total RNA as the amount of template and the following cycling parameters: an initial 30-min incubation at 50°, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Northern blots were performed exactly as described in [28].

The entire coding sequence of all candidate genes was amplified from the resistant strain C57BL/6J and the susceptible strain C3H/HeJ by RT-PCR as described above and was sequenced. A total of 500 ng template DNA was sequenced using ABI Big Dye terminator chemistry (Perkin Elmer) according to the manufacturer's specifications. The reactions were performed in an MJ Research thermal cycler (PTC-225). An ABI 3700 was used for detection. DNA sequences were extracted using ABI Data Collection software (Perkin Elmer).

Phenotype analysis of the CAST/Ei × C57BL/6J F2 intercross

A 44-animal CAST/Ei × C57BL/6J F2 intercross was analyzed in order to map the LeTx susceptibility phenotype in these strains. Macrophages were harvested as stated above and phenotyped as described in [9]. Using the program MAPMAKER [29], LeTx susceptibility maps to D11-Mit90, D11Mit320, and D11Die37 at an LOD score of 7.86 in a 44-animal CAST/Ei × C57BL/6J F2 intercross. LeTx susceptibility in this intercross is linked to no other marker at an LOD score higher than 1.7.

Phylogenetic analysis of Kif1C sequence

The neighbor-joining, distance-based phylogram shown in Figure 2b was constructed using the PAUP 4.0β8 [30] software package, with SPRET/Ei defined as the outgroup. The Kimura 2-paramater calculation was used to generate the distance matrix.

Brefeldin-A treatment

Primary macrophages were harvested as stated above, and approximately 4×10^5 cells were plated in 96-well dishes in BMM. BFA (5 μg/ml) was added to the indicated samples, and cells were incubated for 20 min. PA (1 μg/ml) and 100 ng/ml LF were then added, and the cells were incubated for 4 hr at 37°C. Macrophages were then assayed for viability as described in [9].

Production of retrovirus

To produce the Kif1C^{B6} vector, the C57BL/6J allele of the *Kif1C* cDNA was cloned into the pBABE-MN-IRES-E-GFP vector (gift of Dr. Gary Nolan, Stanford University) derived from the moloney murine leukemia virus (MMLV), where it is linked to an internal ribosome entry site (IRES) to drive the translation of GFP. The retrovirus was produced by transiently transfecting constructs, using lipofectamine (Gibco BRL), into a stable human-derived packaging cell line that allows for the replacement of the retroviral envelope glycoprotein by the vesicular somatitis virus G protein (VSV-G) [31]. This packaging cell line was maintained in DMEM media (GIBCO BRL) containing 25 mM HEPES buffer, 10% fetal calf serum, 200 μM L-glutamine, 10 μg/ml streptomycin, 10 U/ml penicillin, 1 μg/ml tetracycline, 2 μg/ml puromycin, and 0.3 mg/ml G418. After transfection, the virus was obtained by incubating the cells in the following media and collecting the media after 168 hr: DMEM containing 25 mM HEPES buffer, 10% fetal calf serum, 200 μM L-glutamine, 10 μg/ml streptomycin, and 10 U/ml penicillin.

Analysis of transduced macrophages

Primary macrophages were harvested as stated above. Mice were pretreated with 5-fluororuacil (200 mg/kg intravenously) 4 days prior to bone marrow harvest. Two days after bone marrow harvest, the BMM was aspirated, and 10 ml of the previously collected, virus-containing media was added to the cells. Polybrene (Sigma) was also added at a final concentration of 8 μg/ml to increase transduction efficiency. Cells were incubated for 4 hr at 37°C. Macrophages were transduced with the control-GFP construct (C3H/HeJ-GFP) or the Kif1C^{B6} construct (C3H/HeJ-Resistant). The virus-containing media was then aspirated, and the cells were grown for 10 days in BMM. Transduced macrophages were harvested by scraping and were sorted on the basis of fluorescence. Cells expressing GFP were assayed for viability in response to challenge with LeTx. Approximately 1×10^4 cells were plated in 96-well dishes in BMM with 1 μg/ml PA and 100 ng/ml LF for 4 hr at 37°C. Macrophages were then assayed for viability as described in [9].

Analysis of map kinase kinase 3 cleavage

A polyclonal antibody generated against a C-terminal epitope of map kinse kinase 3 (MKK3) was purchased from Santa Cruz Biotechnology (catalog number SC-961). Primary macrophages from C57BL/6J and C3H/HeJ mice, differentiated from bone marrow cells according to published protocols [9, 25], were grown to confluence in 6-well dishes (Costar). Cells were either processed without toxin treatment or after a 1-hr incubation in 10 μg/ml PA and 1 μg/ml LF. Protein extracts were prepared from the cells by resuspending in 200 μl SDS sample buffer (50 mM DTT, 62.5 mM Tris (pH 7.0), 2% w/v SDS, 10% glycerol, and .1% bromophenol blue). Proteins were separated on a 10% SDS-PAGE gel and were transferred to PDVF membranes using a Bio-Rad wet transfer apparatus. Membranes were probed with a 1:3000 dilution of the MKK3 antibody, washed, and processed with the enhanced chemiluminescence detection kit (Amersham Pharmacia) according to the manufacturer's suggested protocol.

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